

Enhancement of the *in Vitro* and *in Vivo* Antitumor Activities of Phosphorylated Mitomycin C and Etoposide Derivatives by Monoclonal Antibody-Alkaline Phosphatase Conjugates

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ABSTRACT

Alkaline phosphatase (AP) was covalently linked to the two antitumor monoclonal antibodies, L6 (anticarcinoma) and 1F5 (anti-B lymphoma), forming conjugates that could bind to antigen-positive tumor cells. The conjugates were able to convert the prodrugs, mitomycin phosphate (MOP) and etoposide phosphate (EP), into an active mitomycin C derivative, mitomycin alcohol, and etoposide, respectively. MOP and EP were less toxic to cultured cells from the H2981 lung adenocarcinoma than their respective hydrolysis products, mitomycin alcohol and etoposide, by a factor >100, and they were also less toxic in mice. Pretreatment of H2981 cells with L6-AP greatly enhanced the cytotoxic effects of MOP and EP, while 1F5-AP caused no such enhancement. A strong antitumor response was observed in H2981-bearing mice that were treated with L6-AP followed 24 h later by either MOP or a combination of MOP and EP. This response was superior to that of MOP or combinations of MOP and EP given alone.

INTRODUCTION

Much attention has been directed towards the use of Mabs² to tumor-associated antigens as vehicles for the delivery of cytotoxic agents to tumors (1-6). The rationale of using such delivery systems is that by carrying the drug to the tumor site, its therapeutic effectiveness will be maintained or increased, while the toxic effects to nontarget tissue will be minimized.

Unfortunately, there are a multitude of problems inherent with this approach. The low potency of many anticancer drugs (2, 3), the heterogeneity of antigen expression (7), and the physiological barriers that prevent uniform distribution of antibodies throughout the tumor mass (8) will lead to a population of tumor cells that are not exposed to cytotoxic quantities of the targeted agent. Another important consideration is that most cytotoxic agents exert their activity inside the cell, requiring that the MAB carrier not only bind to the vast majority of tumor cells but also facilitate the delivery of the cytotoxic agent to its precise site of activity within the cell. It is clear that many MABs against tumor antigens are not capable of doing this (1, 9).

We (10) and others (11) have developed a new method for the delivery of cytotoxic agents in which MABs are used to localize enzymes into the tumor mass. The enzymes are chosen for their ability to convert prodrugs (inactive drug precursors) into active cytotoxic drugs. The approach (Fig. 1) is designed to circumvent the problems of low drug potency, unequal distribution of the conjugate in the tumor mass, and the need to deliver the cytotoxic agent inside the cell, since the drug is

extracellularly released by the enzyme and can then penetrate into nearby tumor cells, many of which may even be inaccessible to the conjugate. We now wish to report the activation of mitomycin and etoposide phosphate derivatives by MAB-AP conjugates. The *in vitro* cytotoxicities and *in vivo* antitumor activities of such conjugate-prodrug combinations are described.

MATERIALS AND METHODS

Materials. Etoposide and mitomycin C were obtained from Bristol-Myers Company, Wallingford, CT. EP was kindly provided by Mark Saulnier (Bristol-Myers). Mitomycin A (12) and MOH (13) were prepared as described previously. The MABs used were L6 (IgG2a), which binds to an antigen on human carcinomas (14), and 1F5 (IgG2a), which is specific for the CD20 antigen on normal and neoplastic B-cells (15). AP from calf intestine was purchased from Calzyme (San Luis Obispo, CA). MAB-AP conjugates (thioether linkage between the proteins) were prepared as described earlier (10). The cell line H2981 was established at Oncogen from a human lung adenocarcinoma. Fluorescence activated cell sorter analysis indicated that L6 binds strongly to H2981 cells, while 1F5 shows very weak binding.

Preparation and Hydrolysis of 9a-Methoxy-7-[(phosphonoxy)ethyl]-amino]mitosane Disodium Salt (MOP). A solution of 2-aminoethyl dihydrogen phosphate (56 mg, 0.4 mmol) in H₂O (0.35 ml) and triethylamine (0.3 ml, 2 mmol) was added to mitomycin A (12) (140 mg, 0.4 mmol) in methanol (6 ml). The reaction was allowed to proceed at room temperature overnight. Saturated aqueous sodium bicarbonate (1.4 ml) was added, and the solution was partitioned between H₂O and methylene chloride. The aqueous phase was concentrated to dryness, and several portions of methanol were added and evaporated. The residue was taken up into methanol and filtered, and the methanol was evaporated. An aqueous solution of the product was applied to a 2- × 10-cm C₁₈ (reverse phase) silica column, and the desired compound was eluted with H₂O. After evaporation of the H₂O, methanol was added and evaporated as before, and the residue was dried for 24 h under high vacuum in a desiccator with phosphorus pentoxide. MOP was obtained as a fine blue powder (190 mg, 97%). The following analytical information was used to confirm the structure: 360 MHz ¹H nuclear magnetic resonance (D₂O) δ 1.94 (s, 3H, CH₃), 2.9-3.1 (m, 4H), 3.20 (s, 3H, OCH₃), 3.28 (s, 1H), 3.36 (s, 1H), 3.5-3.65 (m, 4H), 4.1-4.25 (m, 2H), 4.50-4.57 (dd, 1H, 10-H); high resolution mass spectrum, *m/e* 503.0912 (calculated, 503.0920).

The half-life of MOP (1 mM in 100 mM Tris, pH 7.2) hydrolysis by AP (1 µg/ml) was determined by HPLC using a C₁₈ column (4.6 × 150 mm) and the following gradient conditions: 10 to 60% methanol in 0.1 M NaH₂PO₄ (pH 7.0) over 15 min; 1 ml/min; monitored at 365 nm. The retention times of MOP and MOH were 8.1 and 12.5 min, respectively. The hydrolysis of EP was analyzed by HPLC as described previously (10). HPLC was also used to measure the stability of the drugs (0.1 mM) in human sera, in mouse sera, and in 10% fetal calf sera. Before each analysis, serum proteins were precipitated by adding equal volumes of methanol containing 10 mM EDTA.

***In Vitro* Studies.** H2981 cells in medium (Iscove's modified Dulbecco's medium with 10% v/v fetal calf serum, 200 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine) were plated into 96-well microtiter plates at 10,000 cells/well and allowed to adhere over-

Received 3/7/89; revised 5/25/89; accepted 7/27/89.

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² The abbreviations used are: MAB, monoclonal antibody; AP, alkaline phosphatase; EP, etoposide phosphate; MOP, mitomycin phosphate; MOH, mitomycin alcohol; HPLC, high pressure liquid chromatography; s, singlet; m, multiplet; dd, doublet of doublets.

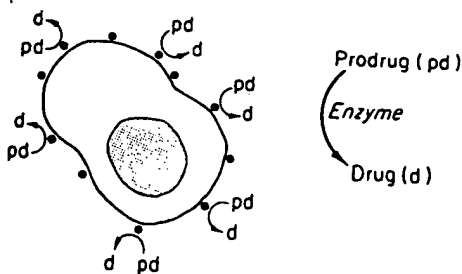


Fig. 1. Activation of prodrugs by targeted enzymes. MAb-enzyme conjugates on the surface of tumor cells convert prodrugs (*pd*) into active cytotoxic drugs (*d*).

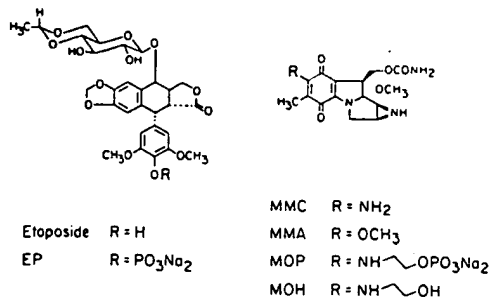


Fig. 2. Structures of etoposide and mitomycin derivatives.

night at 37°C. The cells were washed once and conjugate was added (100 μ l at 10 μ g/ml MAb concentration in medium). After 30 min, the cells were washed 4 times, and the drugs in 50 μ l of medium were added (1 h exposure for the mitomycin series, 5 h exposure for the etoposide series). The cells were washed, 150 μ l of medium were added, and incubation was continued (17 h for mitomycin series, 13 h for etoposide series). [3 H]Thymidine (1 μ Ci in 50 μ l medium) was then added, and after 6 h the cells were frozen to -70°C, thawed, and harvested onto glass fiber discs. The filters were counted on a Beckman 3701 scintillation counter.

In Vivo Studies. Female BALB/c-*nu/nu* mice (4–6 weeks old, from Life Sciences, St. Petersburg, FL) were implanted s.c. with H2981 tumors (approximately 32-mm³ sections from *in vivo* passaging) in the right hind flank. Therapy was initiated in mice with viable, growing tumors 14 days later, once the tumors reached an average size of 100–200 mm³. Conjugates (0.1 ml in PBS which contained 0.25 mg of the antibody component) were injected i.p. according to the schedules in Figs. 4 and 5, and 24 h later 0.2 ml of the drug solutions (6 mg/ml etoposide in 40% dimethyl sulfoxide, 10 mg/ml EP in H₂O, 1 mg/ml MOH in H₂O, 3 mg/ml MOP, or an aqueous mixture of 5 mg/ml EP and 1.5 mg/ml MOP) was injected i.p. Tumor volumes were estimated by the formula

$$\text{Longest length} \times \frac{(\text{perpendicular width})^2}{2}$$

RESULTS

Prodrug and Protein Chemistry. EP was prepared from etoposide as described previously (10). The preparation of MOP involved the displacement of the 7-methoxy group in mitomycin A (12) with 2-aminoethylphosphate (Fig. 2). Both phosphate derivatives were highly water soluble and were rapidly hydrolyzed by AP (calf intestinal, M_r 140,000). The half-lives for the hydrolyses of EP and MOP (at 1.0 mM) by AP (1.0 $\mu\text{g/ml}$) at pH 7.2 were 8 and 12 min, respectively. The identity of the products obtained, etoposide and MOH (13), were confirmed by HPLC comparison with authentic samples.

The prodrugs were also tested for stability in human and mouse sera. The half-life for hydrolysis of EP in either serum at 37°C was approximately 1 h. MOP underwent hydrolysis in

the sera much more slowly than EP. After 4 h at 37°C, only 20% hydrolysis had occurred. Again, HPLC served to establish that the dephosphorylated products, etoposide and MOH, were formed. Both of the prodrugs were stable for at least 5 h in 10% fetal calf serum.

The MAbs L6 (14) and 1F5 (15) were covalently linked to AP through a thioether linkage (10). The chemistry used for conjugation had no apparent effect on the activity of the enzyme or on the binding activities of the antibodies. Fluorescence-activated cell sorter analysis indicated that L6 and L6-AP bound strongly to the H2981 lung adenocarcinoma cell line (saturation at approximately 5 µg/ml), while 1F5 and 1F5-AP bound about 1/100 as well.

***In Vitro* Cytotoxicity.** MOH and etoposide were more cytotoxic to H2981 lung adenocarcinoma than their corresponding phosphorylated derivatives, MOP and EP (Fig. 3). This was determined by measuring the incorporation of [3 H]thymidine into the cellular DNA. MOH (concentration required for 50% cell kill, 1 μ M) was about 100-fold more toxic than the prodrug MOP (10% inhibition at 33 μ M). Similarly, etoposide (concentration required for 50% cell kill, 8 μ M) was much more toxic than EP (15% inhibition at 33 μ M). There was no significant enhancement of the cytotoxic effects of the prodrugs on cells that had been treated previously with 1F5-AP. In contrast, the cytotoxic effects of MOP and EP on H2981 cells that had been treated previously with L6-AP were comparable to those of the active drugs, MOH and etoposide, respectively. These results indicated that the bound conjugate, L6-AP, was capable of converting the prodrugs into active cytotoxic agents.

In Vivo Studies. Before performing *in vivo* therapy studies, preliminary drug toxicology experiments were undertaken. As much as 24 mg/kg/injection of MOP and 120 mg/kg/injection of EP (injected i.p. on days 1 and 5) were tolerated with minimal weight loss. However, using this same treatment protocol, about half as much MOH (12 mg/kg/injection) and etoposide (50 mg/kg/injection) were tolerated. Thus, MOP and EP were less toxic to mice than their respective hydrolysis products, MOH and etoposide.

Therapy experiments were performed on nude mice that had s.c. H2981 tumor xenografts 100–200 mm³ in volume. Treatment was initiated 14 days after tumor implantation by first

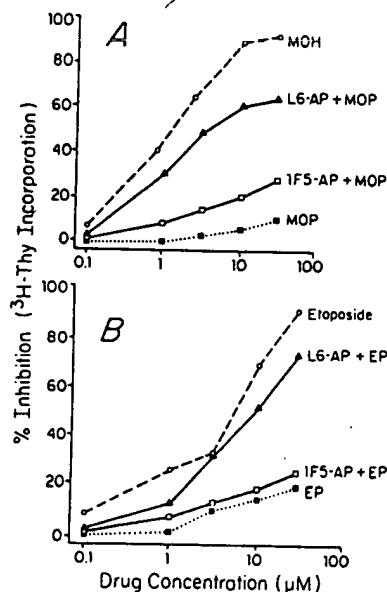


Fig. 3. Cytotoxic effects of (A) MOH, MOP, and MAB-AP conjugates with MOP and (B) etoposide, EP, and MAB-AP conjugates with EP. Thy, thymidine.

injecting the conjugates i.p., followed 24 h later by i.p. treatment with MOP at approximately the maximum tolerated dose. Controls included groups that were untreated or treated with the drugs or prodrugs without prior conjugate administration.

The therapeutic effects of the mitomycin derivatives is shown in Fig. 4. MOH at a cumulative dose of 36 mg/kg had some antitumor activity. Because of the reduced toxicity of MOP, as compared to MOH, larger doses could be administered. A total cumulative dose of 108 mg/kg of MOP resulted in a more pronounced antitumor effect than that seen for animals treated with MOH.

The antitumor activity of MOP was potentiated by prior treatment with either of the two MAb-AP conjugates. Table 1 shows the responses of individual tumors to the therapy. There were complete regressions in 3 of the 6 tumors in mice treated with L6-AP plus MOP as observed at day 63, while no comparable responses were seen in any of the control groups. One of the complete regressions was an apparent long term cure, since this mouse was still tumor free on day 115. Based on the delay in tumor growth, therapy with L6-AP plus MOP resulted in the elimination of >99.99% of the tumor cells (4.3 log cell kill). Treatment with 1F5-AP plus MOP resulted in 3.7 log cell kill, and a smaller therapeutic effect (1.2 log cell kill) was obtained in animals treated with MOH.

A second *in vivo* therapy experiment involved the combination of MOP and EP. Solutions of the prodrugs were combined prior to i.p. injection according to the schedule shown in Fig. 5. The dose of the prodrugs used was one-half of the maximal tolerated dose of each agent. As shown in Fig. 5, it is apparent that while the combination of MOP and EP had significant activity, pretreatment with L6-AP prior to the prodrug administration resulted in a much better antitumor response. By day 63 postimplant, 1 of the 6 tumors in the mice receiving L6-AP

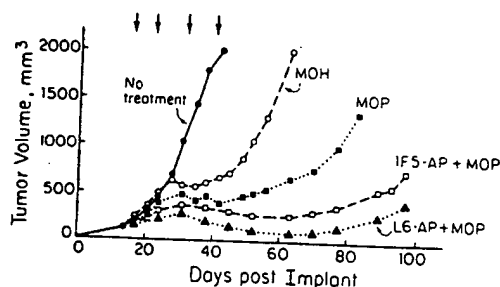


Fig. 4. Effects of MOH, MOP, and conjugates plus MOP on H2981 tumors in nude mice. Arrows, drug treatment schedule. Conjugates were administered 24 h earlier.

Table 1 Effects of various treatments on tumor growth

Treatment group	Response*				Log cell kill ^b
	Progression	Stable	Partial	Complete	
No treatment, day 31	5	0	0	0	
MOH, day 63	6	0	0	0	1.2
MOP, day 63	4	0	0	0	2.3
1F5-AP + MOP					
Day 63	3	2	0	0	3.7
Day 83	4	1	0	0	
L6-AP + MOP					
Day 63	0	3	0	3	4.3
Day 83	3	2	0	1	
MOP/EP, day 63	5	0	0	0	2.0
1F5-AP + MOP/EP, day 63	5	0	0	0	2.0
L6-AP + MOP/EP, day 63	2	3	0	1	3.1

*Progression, continued tumor growth; stable, no additional tumor growth; partial, 50% decrease in size; complete, no apparent tumor.

^bBased on the difference in time required for the treated and nontreated groups to reach a target tumor size of 500–1000 mm³. Tumor doubling time untreated group = 6 days; 1 log cell kill = 3.33 tumor doublings (19.9 days).

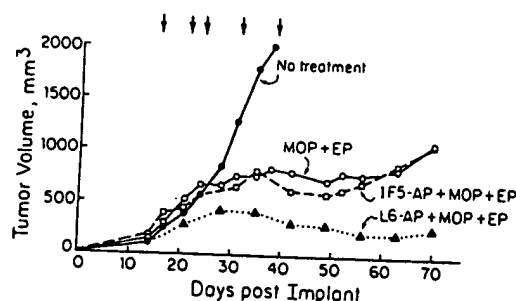


Fig. 5. Effects of MOP plus EP and conjugates plus MOP plus EP on H2981 tumors in nude mice. Arrows, drug treatment schedule. Conjugates were administered 24 h earlier.

plus MOP/EP had undergone a complete regression, and 3 of 6 tumors showed stable responses (Table 1). All of the tumors in mice receiving MOP/EP and 1F5-AP plus MOP/EP underwent progression during this time period.

DISCUSSION

An approach to the use of antibody-enzyme conjugates for the generation of cytotoxic agents was first described in 1973 (16, 17). The targeted enzyme, glucose oxidase, in the presence of lactoperoxidase, glucose, and iodide, caused specific cell death *in vitro*. It is, however, highly unlikely that this strategy could have an *in vivo* antitumor effect, since four separate agents are required to generate a cytotoxic substance. We have reported a much more direct method, in which MAb-AP conjugates were used to convert EP into the clinically approved anticancer drug, etoposide (10). Although AP is present in many biological tissues, a significant antitumor effect, both *in vitro* and *in vivo*, was observed with L6-AP in combination with EP. This paper extends the methodology to include prodrugs of both etoposide and mitomycin and shows that antitumor activity against a lung adenocarcinoma line can be achieved by MAb-AP conjugates together with MOP or a combination of EP and MOP.

MOP was developed as a prodrug for AP because it was known that the product upon hydrolysis, MOH, was as active as mitomycin C in an animal tumor model. MOH has also been reported as being well tolerated at 4 times the dose of mitomycin C (13). As expected, both EP and MOP were much less cytotoxic against H2981 *in vitro* than etoposide and MOH, respectively, and they were also less toxic *in vivo*. It was possible to administer 3 times as much MOP as MOH using the treatment schedule shown in Fig. 4. The antitumor effect of MOP was greater than that of MOH, possibly owing to a number of factors including the increased amount which could safely be injected, alterations in its biodistribution, and the fact that endogenous phosphatases can convert MOP to MOH.

Both the binding conjugate, L6-AP, and the nonbinding conjugate, 1F5-AP, significantly increased the *in vivo* antitumor activity of MOP (Fig. 4). The slightly greater activity of L6-AP plus MOP over 1F5-AP plus MOP was most evident at day 63 postimplant, in which 3 of 6 tumors had undergone complete regressions. The fact that 1F5-AP was able to affect any such increase at all was most likely due to insufficient clearance of the conjugate and perhaps to nonspecific uptake of the conjugate by the tumor (18). A greater degree of specificity may be obtained by prolonging the time interval between conjugate and prodrug administration, by altering the doses of prodrug and conjugate used, or by using F(ab')₂ conjugates that may clear from the blood more quickly (19). Studies are currently under way to test these parameters.

One of the advantages of targeting AP is that the enzyme may be capable of hydrolyzing many different phosphorylated prodrugs, thus enabling the use of several prodrugs in combination chemotherapy. This is exemplified in Fig. 5, which shows that L6-AP strongly increases the antitumor activity of MOP and EP given together while 1F5-AP caused no such enhancement in the activity of the two prodrugs. The finding that immunological specificity was observed in the experiment involving coadministration of MOP and EP might be due to the lower drug dose used which would lead to a diminished contribution to the antitumor effect by systemically released drug.

This study demonstrates that a very high degree of *in vivo* antitumor activity (>99.99% cell kill) can be obtained through the combination of MAb-AP conjugates and prodrugs that are activated by the targeted enzyme. In the example reported earlier (10), this effect was immunologically specific and was attributed to the fact that the enzyme localized within the tumor converted the prodrug EP into etoposide. A similar conclusion, although without the use of a nonbinding control antibody, was drawn by another group using a MAb-carboxypeptidase G-2 conjugate in combination with a nitrogen mustard prodrug (11).

The data reported in this paper suggest that both intratumoral and systemic drug activations contribute to the antitumor effects of MAb-enzyme/prodrug therapy and that both may be important for therapeutic efficacy. While a number of important questions concerning the precise mechanism of activity remain to be clarified, it is evident from the activities seen that MAb-enzyme/prodrug therapy has considerable clinical potential.

ACKNOWLEDGMENTS

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